DR. SANCY A LEACHMAN (Orcid ID : 0000-0001-9140-0648)

PROF. DOROTHY CATHERINE BENNETT (Orcid ID : 0000-0002-3639-7527)

PROF. ZALFA ABDEL-MALEK (Orcid ID : 0000-0002-2651-1533)

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In Vitro Behavior and UV response of melanocytes derived from carriers of *CDKN2A* mutations and *MC1R* variants

Barbara Hernando[1], Viki B. Swope[2], Steven Guard[2], Renny J. Starner[2], Kevin Choi[2], Ayesha Anwar[2], Pamela Cassidy[3], Sancy Leachman[3], Ana Luisa Kadekaro[2], Dorothy C. Bennett[4], Zalfa A. Abdel-Malek[2*]

[1]Department of Medicine, Jaume I University of Castellon

Ave. Sos Baynat s/n, 12071, Castellon, Spain

[2]Department of Dermatology, University of Cincinnati

231 Albert Sabin Way, Cincinnati, OH 45267, U.S.A.

[3]Department of Dermatology, Oregon Health and Sciences University

3181 SW Sam Jackson Park Road, Portland, OR 97239, U.S.A.

[4]Molecular & Clinical Sciences Research Institute, St George's, University of London

Box J2A, London SW17 0RE, UK

Correspondence:
Zalfa Abdel-Malek, Department of Dermatology, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, OH, 45267, U.S.A.
Tel: 513-558-6246
Fax: 513-558-0198
Email:abdelmza@ucmail.uc.edu

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SUMMARY:

Co-inheritance of germline mutation in *cyclin-dependent kinase inhibitor 2A* (*CDKN2A)* and loss-of-function (LOF) *melanocortin 1 receptor* (*MC1R*) variants is clinically associated with exaggerated risk for melanoma. To understand the combined impact of these mutations, we established and tested primary human melanocyte cultures from different *CDKN2A* mutation carriers, expressing either wild-type *MC1R* or *MC1R* LOF variant(s). These cultures expressed the *CDKN2A* product p16 (INK4A) and functional MC1R. Except for 32ins24 mutant melanocytes, the remaining cultures showed no detectable aberrations in proliferation or capacity for replicative senescence. Additionally, the latter cultures responded normally to ultraviolet radiation (UV) by cell cycle arrest, JNK, p38, and p53 activation, hydrogen peroxide generation, and repair of DNA photoproducts. We propose that malignant transformation of melanocytes expressing *CDKN2A* mutation and *MC1R* LOF allele(s) requires acquisition of somatic mutations facilitated by *MC1R* genotype or aberrant microenvironment due to *CDKN2A* mutation in keratinocytes and fibroblasts.

Abbreviations: CDKN2A=Cyclin-dependent kinase inhibitor 2 A; Rb=Retinoblastoma protein; MC1R=Melanocortin 1 receptor; LOF=Loss of function; α-MSH=α-Melanocyte stimulating hormone; UV=Ultraviolet radiation; cAMP=Cyclic adenosine monophosphate; CDK=Cyclin-dependent kinase; CPD=Cyclobutane pyrimidine dimers

SIGNIFICANCE:

Epidemiological evidence shows that co-inheritance of germline *CDKN2A* mutation and loss-of-function *MC1R* variant exacerbates the risk for melanoma above that resulting from mutation in either gene. To investigate the mechanism for this increased risk, we established primary human melanocyte cultures from *CDKN2A* mutation carriers who expressed either wild-type or LOF *MC1R* variant(s). Except for 32ins24 *CDKN2A* mutation, we found no detectable differences in the *in vitro* behavior or response to UV of these melanocytes. We propose that co-inheritance of *CDKN2A* and *MC1R* mutations exaggerates melanoma risk by promoting the mutator phenotype of melanocytes possibly by an aberrant cutaneous microenvironment.

Key words:
CDKN2A, MC1R, proliferation, replicative senescence, ultraviolet radiation

INTRODUCTION:

Two important melanoma predisposition genes are *CDKN2A* and melanocortin 1 receptor gene (*MC1R).* Germline mutations in *CDKN2A* are the most prevalent in familial melanoma pedigrees, and

also increase the risk for other malignancies, such as pancreas, lung, and breast cancers (de Snoo et al., 2008; Goldstein et al., 2006). These mutations occur in less than 2% of all melanoma cases, yet are highly penetrant, increasing the lifetime risk of melanoma by 67% (Bishop et al., 2002). Loss-of-function variants of *MC1R* (namely R151C, R160W, and D294H, designated as R alleles), associated with red hair phenotype, poor tanning ability and increased risk for melanoma, are more common but less penetrant than *CDKN2A* mutations (Box, Wyeth, O'Gorman, Martin, & Sturm, 1997; Kadekaro et al., 2010; Smith et al., 1998). Twenty-four percent of melanoma patients carry *MC1R* LOF variants, and although redheads who express two *MC1R* LOF alleles comprise only 1-2% of the worldwide population, they represent 16% of all melanoma patients (Olsen, Carroll, & Whiteman, 2010; Williams, Olsen, Hayward, & Whiteman, 2011).

CDKN2A and *MC1R* serve important functions to preserve the genomic stability of melanocytes. The tumor suppressor p16 INK4A (p16), one product of the *CDKN2A* locus, is a cyclin-dependent kinase (CDK) 4/6 inhibitor, which causes G1 arrest by activating the retinoblastoma (Rb) protein and suppressing the transcription factor E2F1 (Chicas et al., 2010; Sun, Bagella, Tutton, Romano, & Giordano, 2007). An important function of p16, particularly in melanocytes, is regulation of senescence (Fung, Pupo, Scolyer, Kefford, & Rizos, 2013; Haferkamp et al., 2009; Rayess, Wang, & Srivatsan, 2012; Sviderskaya et al., 2003). Additionally, mutations in *CDKN2A* seem to reduce nucleotide excision repair capacity and contribute to oxidative stress (Jenkins et al., 2011; Sarkar-Agrawal, Vergilis, Sharpless, DePinho, & Runger, 2004) . The *MC1R* codes for a G_s protein-coupled receptor that is expressed on the cell surface of melanocytes (Abdel-Malek et al., 2014; Abdel-Malek et al., 1995). Activation of the MC1R by its agonist α-melanocyte stimulating hormone (α-MSH) stimulates eumelanin synthesis, reduces ultraviolet radiation (UV)-induced generation of reactive oxygen species and enhances DNA repair in human melanocytes (Kadekaro et al., 2010; Song et al., 2009; Swope et al., 2014). An additional mechanism by which the activated MC1R prevents the genotoxic effects of UV is by inhibiting the degradation of the tumor suppressor PTEN, thereby leading to inactivation of the phosphatidylinositol 3 kinase/Akt oncogenic pathway (Cao et al., 2013).

Multiple epidemiological studies reported that co-inheritance of a mutant *CDKN2A* and *MC1R* LOF variant exacerbates melanoma risk and results in early onset of melanoma (Box et al., 2001; Demenais et al., 2010; Fargnoli, Gandini, Peris, Maisonneuve, & Raimondi, 2010; Goldstein et al., 2005). *CDKN2A* mutation carriers with one *MC1R* variant showed a double melanoma risk as compared to *CDKN2A* mutation carriers expressing wild type *MC1R* (Fargnoli et al., 2010). This led us to compare the *in vitro* behavior of primary human melanocyte cultures established from skin biopsies of members of three familial melanoma kindreds who are carriers of one of 3 known deleterious *CDKN2A* mutations, and expressing either wild type or LOF *MC1R* variant(s), and of those established from donors

expressing wild-type for *CDKN2A* and similar *MC1R* genotype (Table 1). We focused on investigating cultured melanocytes, given that they are the skin cells most drastically affected by *CDKN2A* mutations or *MC1R* variants. The endpoints we examined were: expression of p16, INK4B (p15), and Rb proteins, proliferation rate, capacity for replicative senescence, and various aspects of the DNA damage response to UV. *In vitro*, melanocytes heterozygous for V126D or 5'UTR-34G>T mutation, even those carrying *MC1R* LOF allele(s), were similar to their *CDKN2A* wild-type counterparts in all these parameters. Our results confirm that malignant transformation of *CDKN2A* mutant and *MC1R* variant melanocytes requires accumulation of additional "hits", such as somatic mutations in genes that regulate proliferation or apoptosis, as reported (Bennett, 2016). Expression of *MC1R* LOF variants increases the chance for somatic mutations that drive melanomagenesis (Robles-Espinoza et al., 2016). It is also conceivable that *CDKN2A* germline mutations in keratinocytes and fibroblasts result in an aberrant microenvironment that promotes melanocyte transformation. Our results underscore the importance of investigating the impact of co-inheritance of *CDKN2A* and *MC1R* mutations on melanocytes in the context of the skin as an organ. We have established from the same cohort primary keratinocyte and fibroblast cultures that we will use to investigate the impact of *CDKN2A* genotype on their secretome, and to develop 3D skin models to address how the cutaneous microenvironment might affect the genomic stability of melanocytes.

MATERIALS AND METHODS:

Establishment of primary melanocyte cultures and UV irradiation:

Primary adult human melanocyte cultures were established from skin biopsies of members of familial melanoma kindreds, or other patients of the Huntsman Cancer Institute Melanoma Clinic in Utah, after informed consent (IRB protocol; # 7916). Other cultures were established from surgical discards of adult skin or from neonatal foreskins. All of these cultures were maintained in our previously described routine melanocyte growth medium (Abdel-Malek et al., 1995). However, the cultures 13a and 14a failed to proliferate in this medium, and required a more enriched growth medium in order to replicate, as described earlier (Sviderskaya et al., 2003). The enriched medium consisted of MCDB 153, 2% fetal bovine serum, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 28 µg/ml bovine pituitary extract, 1 nM endothelin-1, and 1 ng/ml basic fibroblast growth factor. The cultures were authenticated by immunostaining for the melanocyte-specific marker TRP-1. Penicillin/streptomycin and antimycotics, as well as plasmocin, were added routinely to the culture media. For experiments involving UV exposure, melanocytes were irradiated with a dose of 75 or 90 mJ/cm^2 UV, as described (Kadekaro et al., 2010).

cAMP measurement:

The levels of cAMP were determined using a radioimmunoassay, as described (Suzuki, Cone, Im, Nordlund, & Abdel-Malek, 1996).

Determination of doubling time:

Melanocytes were plated in 6-well plates at a density of $0.15x10^6$ cells/well (day 0). To determine the effect of UV on proliferation, melanocytes were irradiated with a sublethal dose of 75 mJ/cm^2 UV on day 3. Medium was changed, and cell numbers in the control and UV-irradiated groups were determined on day 3, and every other day thereafter, for 10 more days (n=3 wells/time point). The doubling times were calculated from the slope of the growth curve.

Cell cycle analysis:

Melanocytes were plated at a density of $0.3x10^6$ cells/60 mm dish. On day 3 after plating, cells were irradiated with 0 (control) or 75 mJ/cm^2 UV, and fresh medium was added thereafter. Cell cycle analysis was performed at 0, 6, 24, 48, and 72 h after UV as described (von Koschembahr, Swope, Starner, & Abdel-Malek, 2015).

β-galactosidase staining:

Melanocytes were plated at a density of $2x10^4$ cells/well in an 8-well chamber slide (4 wells/group). After 3 days, cells were stained using a commercially available kit (Cell Signaling). Microscopic images were acquired, the percent of β-galactosidase positive melanocytes were quantified, and the data expressed as mean ± SEM. At least 500 melanocytes were analyzed/group.

Quantitation of cyclobutane pyrimidine dimers (CPD):

Melanocytes were plated at $0.3x10^6$ cells/60 mm dish, and irradiated 3 days thereafter with 0 (control) or 90 mJ/cm^2 UV, a dose that results in extensive DNA damage, but moderate apoptosis (≤20%). At least triplicate dishes were included in each group. In some experiments, melanocytes were treated with α-MSH prior to, and post UV-irradiation, as described (Kadekaro et al., 2010). Three, and 48 h post UV, melanocytes were harvested and fixed, immunostained with CPD-specific antibody (CosmoBio), and analyzed by flow cytometry, as described (Kadekaro et al., 2010).

Measurement of hydrogen peroxide:

Melanocytes were plated and irradiated with 90 mJ/cm^2 UV. Generation of hydrogen peroxide was determined at 0, 30, and 60 min post UV as described (Song et al., 2009).

Western blot analysis:

For detection of p16, p15, and p-Rb, melanocytes were plated at a density of $0.75\text{-}1x10^6$ in 100 mm dishes. Three days thereafter, fresh medium was added, and 24 h later, cells were lysed using RIPA buffer containing protease and phosphatase inhibitors. Western blotted proteins were detected using specific antibodies for p16 or p15 (Abcam; Cambridge, MA), and p-Rb (Cell Signaling, Danvers, MA). For detection of p- and total JNK and p38, p-Rb, p53 and GADD45α, melanocytes were plated as above, irradiated with 0 (control) or 75 mJ/cm^2 UV, and cell lysates prepared at 0.5,1.5, 4, 6, and 24 h. Phospho-

and total JNK and p38 antibodies were purchased from Cell Signaling, and p53 and GADD45α antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX).

RESULTS AND DISCUSSION:

Expression of p16, Rb, and p15, and MC1R activity in cultured melanocytes:

We established primary human melanocyte cultures from members of three familial melanoma pedigrees who are heterozygous for the *CDKN2A* mutation V126D and expressing either wild-type or *MC1R* LOF variant R151C, R160W, and D294H, or heterozygous for *CDKN2A* 5'UTR-34G>T or 32ins24 mutation and LOF *MC1R* variant, or V60L, a variant associated with blonde hair and modest increase in melanoma risk (Box et al., 1997; Kadekaro et al., 2010; Smith et al., 1998). The *CDKN2A* V126D mutation in exon 2 creates a temperature-sensitive protein that binds to CDK4 efficiently at the permissive temperature of 34^{o}C (Goldstein et al., 2001; Parry & Peters, 1996). The 5'UTR-34G>T mutation in exon 1 introduces an alternative translational start site that was reported to reduce expression of the wild-type protein (Liu et al., 1999). The 32ins24 mutation in exon 1 codes for a protein containing 8 additional amino acids (Eliason et al., 2006). These mutations are deleterious, and do not affect expression of p14 ARF, the second *CDKN2A* product (Goldstein et al., 2006). For all experiments, with exception of 13a and 14a, all cultures were maintained in the same routine melanocyte growth medium (Abdel-Malek et al., 1995).

Except for very rare cases worldwide, all *CDKN2A* familial melanoma patients are heterozygous for a *CDKN2A* mutation, yet the normal allele can be epigenetically silenced, resulting in loss of heterozygosity (Castellano & Parmiani, 1999). We first investigated the expression of p16 protein, the product of *CDKN2A*, in the melanocyte cultures at early (≤8) versus late (≥9) passage, to determine the impact of each of the three *CDKN2A* mutations and possibly LOF *MC1R* variants, and to rule out epigenetic silencing over time in culture. By Western blot analysis, we detected p16, and phospho (p)-Rb, the inactive form of Rb, given that p16 activates Rb by inhibiting its phosphorylation, in a panel of melanocyte cultures listed in Table 1. Controls were melanocyte cultures expressing wild-type *CDKN2A* and *MC1R*, or a *MC1R* LOF allele, as well as two previously described rare melanocyte cultures, 13a (Hmel-16-1) and 14a (Hmel-16-II) with two mutant *CDKN2A* alleles (Gruis et al., 1995; Sviderskaya et al., 2003). As reported earlier, 13a lacked p16, while 14a expressed high p16 levels (Fig. 1) (Gruis et al., 1995; Huot et al., 2002). The remaining cultures expressed p16 at different levels, and *CDKN2A* 32ins24 mutation carriers 11a and 12a, expressed p16 as two bands, with the higher molecular weight band representing the product of the mutant allele (Fig. 1). The levels of p16 protein did not correlate with *MC1R* genotype. Expression of p16 remained detectable in late passage melanocytes (Fig. 1b), which approached replicative senescence, as depicted by marked prolongation of doubling time and increase in

the percent of β-galactosidase-positive melanocytes (Fig. 2, 3). These results suggest that *CDKN2A* is not epigenetically silenced with increased passage in culture.

The level of p-Rb, which is reduced by p16, varied among the different cultures, possibly due to differences in their proliferation rate, passage number, and overall genetic background (Fig. 1a). In early-passage melanocytes, 24 h after addition of fresh growth medium, the levels of p-Rb induced by mitogens, and representing inactive Rb, were highest in 3a and 5a, and in p16-null 13a melanocytes (Fig. 1a). There seemed to be an inverse correlation between p16 and p-Rb levels, particularly in late passage cultures (Fig. 1b, Fig. S1). The levels of p-Rb were lowest in late-passage cultures 11a, 12a, 14a, 15a, 20a, and 23a with the highest levels of p16, and low or no proliferation capacity, as depicted below in Fig. 1b, 2, 3, and Fig.S1. Late passage p16-null 13a also expressed very low levels of p-Rb, possibly due to the inhibitory effect of other cyclin/cdk inhibitors. These results suggest that p16 mutation carriers continue to express functional p16 protein and/or other cyclin/cdk inhibitors with increased passage in culture.

Similar to p16, p15, the product of *CDKN2B*, adjacent to *CDKN2A*, functions as a cyclin-D-CDK4/6 inhibitor, and promotes melanocyte senescence (Hannon & Beach, 1994; McNeal et al., 2015). We examined the levels of p15 in the above melanocyte cultures (Fig. 1b) (McNeal et al., 2015). The p16-null 13a expressed the highest levels of p15. Densitometry analysis revealed an inverse correlation between p16 and p15 levels in 7 of 20 (35%) cultures, mostly in 15a and 23a followed by 3a, 18a and 25a, which expressed the highest levels of p16 and low levels of p15, and in 19a and 21a, which expressed very low levels of p16 and relatively high levels of p15 (Fig. S1), suggesting a compensatory role of these two CDK inhibitors.

To elucidate if *CDKN2A* mutations might reduce MC1R activity, we measured the increase in the levels of cyclic adenosine monophosphate (cAMP), the second messenger for MC1R, in response to treatment with its agonist α-MSH (Suzuki et al., 1996). Melanocytes expressing V126D or 5'UTR-34>T mutation, even those co-expressing a LOF *MC1R* allele, responded to 10 nM α-MSH with significant increase in cAMP levels, which differed among cultures due to donor variability and differential expression of genes that affect *MC1R* expression or its cAMP pathway, as reported earlier (Table S1) (Kadekaro et al., 2010). We have reported that treatment of melanocytes expressing functional MC1R with α-MSH enhanced their capacity to repair UV-induced CPD, the major form of DNA photoproducts (Kadekaro et al., 2010). We found that V126D mutant melanocytes, similar to their wild-type counterparts, responded to α-MSH with enhanced repair of UV-induced CPD, further confirming that the *CDKN2A* mutations investigated did not inhibit MC1R activity (Fig. S2).

Comparison of population doubling time and capacity for replicative senescence of different melanocyte cultures:

Given the significance of p16 as a CDK inhibitor and regulator of senescence, and MC1R signaling in regulating melanocyte proliferation (Sun et al., 2007; Swope, Medrano, Smalara, & Abdel-Malek, 1995), we compared the proliferation capacity of the melanocyte cultures as a function of passage in culture by determining their respective doubling times. In general, almost all melanocytes showed gradual reduction in their proliferation rate, evidenced by prolonged doubling time with increased passage number (Fig. 2). Exceptions were11a and 12a, co-expressing 32ins24 *CDKN2A* mutation and a LOF *MC1R* variant that had the lowest proliferation capacity and longest doubling times even at early passage (5 and 4, respectively) (Fig. 2), which limited their use for further experiments. In contrast, 21a maintained a high proliferation rate and the same doubling time (4.1 days) at passages 9 and 13 (Fig. 2). The doubling time did not seem to correlate with donors' age. For example, at early passages (3-7), the doubling times of 6a and 17a, from donors age 42 and 43, were comparable to those of 8a and 22a, and shorter than that of 18a, from donors in their twenties (Fig. 2, Table 1). The two cultures 13a and 14a, expressing two mutant *CDKN2A* alleles, failed to proliferate in the routine melanocyte growth medium and required a highly enriched growth medium in order to proliferate, as previously reported (Sviderskaya et al., 2003). In our enriched medium, the doubling time of 13a increased from 5 days at passage 14 to 16.8 days at passage 21, and that of 14a was maintained at 11.7 days at passages 17 and 20 (Fig. 2). Unfortunately, early passages of these two cultures are not available. As reported (Fung et al., 2013; Sviderskaya et al., 2003), p16-null 13a exhibited reduction of proliferation rate under highly enriched culture conditions, albeit at a higher passage than their heterozygous or wild-type counterparts, suggesting that p15, and possibly other CDK inhibitors, compensate for p16 loss and drive the senescence program.

The cultures varied in their doubling times independently of *CDKN2A* or *MC1R* genotype, with the exception of 11a and 12a (Fig. 2), and this was corroborated by cell cycle analysis (Table S2). These variations are due to differential expression of other genes that regulate proliferation rate in different donors. Interestingly, the two wild-type cultures 17a and 18a, but not 15a, underwent a sharp decline in proliferation by passages 7 and 9, respectively. The number of population doublings of the different melanocyte cultures until they became quiescent ranged between a minimum of 8-11 for10a -12a, and 18a, to ≥19 for 2a-8a, 15a, and 21a (Table S3), which correlated in most cases with their early doubling time. These results suggest that expression of V126D or 5'UTR-34G>T *CDN2A* mutation, with or without LOF allele did not have an obvious impact on the proliferative capacity of melanocytes, known to be regulated by the crosstalk of multiple signaling pathways (Swope et al., 1995).

Senescence is a state of permanent growth arrest that is reached after cells exhaust their proliferation capacity, and is primarily regulated by p16 in melanocytes (Haferkamp et al., 2009; Sviderskaya et al., 2002). In light of the results in Fig. 2, we investigated whether the marked reduction in

proliferation with passage in culture was due to replicative senescence. For that, we compared the percent of senescent melanocytes at different passages, using β-galactosidase staining (Fig. 3) (Dimri et al., 1995). Consistent with the doubling time data (Fig. 2), the percent of β-galactosidase-positive melanocytes and the intensity of staining increased with passage in culture, regardless of their *CDKN2A* or *MC1R* genotype. An exception was 21a, which had less than 12% of melanocytes positive for β-galactosidase at passage 9 (data not shown), consistent with their persistently high proliferation rate (Fig. 2). Increased β-galactosidase staining was even evident in 13a and 14a, albeit at a much higher passage than other cultures. The percent of β-galactosidase-positive cells increased by about 30% in 13a and 14a from passages 18 and 17 to passages 22 and 21, respectively, further confirming that other effectors, such as p15 and p21 can compensate for loss of p16 function (Fung et al., 2013; Sviderskaya et al., 2003). Even neonatal melanocytes (26n and 29n) showed a remarkable increase in β-galactosidase-positive staining with increased passage (22 and 14, respectively) in culture. We conclude that carriage of mutations in *CDKN2A* even with co-expression of a *MC1R* LOF variant does not abrogate replicative senescence or extend the proliferative capacity of melanocytes.

DNA damage response to UV:

Solar UV is a main etiological factor for melanoma, and UV signature mutations are evident in melanoma tumors (Hodis et al., 2012). The role of MC1R in the UV response of melanocytes is well-documented, and the possible role of p16 in regulating DNA repair has also been reported (Abdel-Malek et al., 2014; Cao et al., 2013; Sarkar-Agrawal et al., 2004). To investigate the DNA damage response to UV of melanocyte cultures with different *CDKN2A* and *MC1R* genotypes (in Table 1), we compared their responses to irradiation with 75 mJ/cm^2 UV, a sub-lethal dose that induced substantial DNA photoproducts . DNA damage induced by UV results in cell cycle arrest to allow for DNA repair (Kastan, Onyekwere, Sidransky, Vogelstein, & Craig, 1991; Lo et al., 2005); Barker et al., 1995). All cultures tested, regardless of genotype, including 13a and 14a, responded to the above UV dose with prolonged doubling time, due to transient G1 and G2 arrest (Fig. 4, Table S2), suggesting their ability to sense DNA damage.

Since V126D is a common *CDKN2A* mutation in melanoma-prone families in the U.S.A. (Goldstein et al., 2001), we further compared the DNA damage response to UV of 4a and 5a to that of 13a, 17a, and 21a, using Western blot analysis for the stress-activated MAP kinases JNK and p38, p53 and its downstream target GADD45α, all known to be activated by UV, and p-Rb, which is inhibited by UV (Fig. 5, Fig. S1) (Medrano, Im, Yang, & Abdel-Malek, 1995; M. L. Smith et al., 1996; Tada, Pereira, Beitner-Johnson, Kavanagh, & Abdel-Malek, 2002). All five cultures had similar responses, regardless of their genotype. Figure 5a, b, and c represent the responses of 5a, and Fig. S3 represents those of 13a. Phosphorylation, i.e. activation, of the stress-induced MAP kinases JNK and p38, was evident as early as

30 min post UV, and increased for at least 4 h (Fig. 5a). The p53 protein increased time-dependently for at least 24 h, beginning 3 h post UV (Fig. 5b). The levels GADD45α also increased in response to UV, indicating p53 transactivation (Fig. 5c, Fig. S3). In the control unirradiated groups, addition of fresh medium increased the levels of p-Rb time-dependently, indicative of Rb inactivation by mitogens, while irradiation with UV reduced p-Rb, consistent with cell cycle arrest (Fig. 4, Fig. 5b). The increase in p-Rb in the unirradiated control groups was markedly less in the late, as compared to early passage cultures, consistent with reduced proliferation in the former (Fig 5b). These findings demonstrate that mutations in *CDKN2A*, even complete loss of p16, do not inhibit the above responses of melanocytes to UV.

The DNA damage response to UV involves activation of DNA repair to maintain genomic stability (Cimprich & Cortez, 2008). We compared repair of CPDs in 2a, 5a, 7a, 13a, and 16a, and found substantial DNA repair, with only 28-44% of mean fluorescence of CPDs remaining at 48 h, as compared to 3 h post UV (Fig. 5d). These results further confirm that a mutant*CDKN2A* allele, even lack of p16 and co-expression of a *MC1R* LOF variant, does not impair the capacity of melanocytes to repair DNA photoproducts.

Irradiation of melanocytes with UV results in the rapid generation of hydrogen peroxide (Song et al., 2009). Previous reports indicated that p16 is critical for reduction of oxidative stress ((Jenkins et al., 2011). However, exposure of melanocytes with different *CDKN2A* and *MC1R* genotypes to 90 mJ/cm^2 UV resulted in increased levels of hydrogen peroxide, which reached a maximum at 60 min in all the cultures tested (Table S4). The variability in basal and UV-induced hydrogen peroxide levels among the cultures with different *CDKN2A* or *MC1R* genotype can be attributed to donor variability and differential expression of genes that regulate the antioxidant defenses.

In summary, using primary melanocyte cultures that naturally express a *CDKN2A* mutation with or without a *MC1R* LOF allele, and multiple functional assays, we found that these melanocytes do not markedly differ in their *in vitro* behavior from their wild-type counterparts, and respond appropriately to a single dose of UV. These findings confirm that malignant transformation of *CDKN2A* mutant melanocytes requires additional hits, such as repetitive UV exposure, loss of p15, or somatic mutations facilitated by expression of *MC1R* LOF variant(s) (Robles-Espinoza et al., 2016). It would have been very informative to compare the genome of melanoma tumors from the same mutation carriers to that of their premalignant melanocytes, but none of those carriers had a melanoma at the time of skin biopsy. Based on the reports that *CDKN2A* germline mutations alter global gene expression in fibroblasts and keratinocytes (Fan et al., 2013; Puig-Butille et al., 2014), it is conceivable that these mutations, which are not exclusively melanocyte-autonomous, result in an aberrant microenvironment, which is conducive for genomic instability of melanocytes. Fortunately, we established primary keratinocyte and fibroblast cultures from the biopsies of donors listed in Table 1. This will enable us to conduct future experiments to

determine the impact of *CDKN2A* mutations on the secretome of keratinocytes and fibroblasts, and to investigate the interactions of these cells with melanocytes in the context of a 3D cultured skin model.

FIGURE LEGENDS:

Figure 1. Detection of p16, p-Rb, and p15 in melanocytes with different *CDKN2A* and *MC1R* genotypes using Western blot analysis. In (a), p16 and p-Rb were detected in early-passage (≤8) cultures. Bands 1a-4a, 7a, 8a, 11a, 12a, 17a, and 18a were detected on the same membrane; 5a, 9a, 10a, and 21a were detected on a second membrane, and 6a, 13a, 14a, and 23a were detected on a third membrane. In (b), p16, p-Rb and p15 were detected in late-passage (≥9) cultures. Actin was used as loading control. +/+= wild-type; -/-= expression of 2 mutant *CDKN2A* alleles; R/+=heterozygous for a red hair (R) LOF *MC1R* allele; R/R=homozygous or compound heterozygous for 2 R LOF *MC1R* alleles; R/r= compound heterozygous for a LOF variant and the moderate V60 allele; p/+= heterozygous for the pseudoallele V92M *MC1R* variant.

Figure 2. Effect of passage in culture on the doubling time of melanocyte cultures with different *CDKN2A* and *MC1R* genotypes. The star symbol denotes that the calculated doubling time exceeded 55 days.

Figure 3. Comparison of the percent of β-galactosidase-positive melanocytes with different *CDKN2A* and *MC1R* genotypes at early versus late passage in culture. Each bar represents the mean of at least 500 melanocytes ± SEM. *= Statistically different from the earlier passage(s) at $p<0.05$, as determined by ANOVA, followed by SNK. The upper panels represent light microscopic views of 15a, as they proliferated at a high rate in early passage (in a), and when they reached replicative senescence at passage 17 (in b).

Figure 4. Effect of UV exposure on the doubling time of melanocyte cultures with different *CDKN2A* and *MC1R* genotypes. Melanocytes were irradiated with UV and the doubling time determined as described in Materials and Methods. The star symbol denotes that melanocytes failed to show significant recovery from the UV-induced growth arrest by the end of the experiment (10 days post UV).

Figure 5. Expression of p-JNK, p-p38, p-Rb, p53, and GADD45α in response to UV, as depicted by Western blot analysis, and the repair of CPD, by melanocytes with different *CDN2A* and *MC1R* genotypes. The presented data were obtained using cell lysates derived from 5a melanocytes. Panel (a) represents the time-dependent expression of p- and total JNK and p- and total p38. Panels (b) and (c) represent expression of p53, p-Rb, and GADD45α in early- vs. late- passage (6 and 13, respectively).

Vinculin and actin were used as loading controls. Similar results were obtained with 4a, 13a, 17a and 21a. (d) represents induction (3h) and repair (48 h post UV) of CPD in melanocytes with different genotypes. Each data point is the mean of 3-5 determinations ± SEM. *= Statistically different from the respective 3 h time point at $p<0.05$, as determined by ANOVA followed by SNK.

CONFLICT OF INTEREST:

All authors declare no conflict of interest

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Cell Strain	Age	Gender	*CDKN2A* Genotype	*MC1R* Genotype
1a	28	M	V126D/+	+/+
2a	20	F	V126D/+	+/+
3a	31	F	V126D/+	D294H/+
4a	18	M	V126D/+	D294H/+
5a	35	F	V126D/+	R160W/+
6a	42	M	V126D/+	R160W/R151C
7a	24	M	5'UTR-34G>T/+	D294H/+
8a	21	M	5'UTR-34G>T/+	R160W/+
9a	18	M	5'UTR-34G>T/+	R160W/+
10a	22	M	5'UTR-34G>T/+	V60L/T314T
11a	32	F	32ins24/+	R160W/V60L
12a	22	M	32ins24/+	R160W/+
13a	?	M	266_244del19	+/+
14a	?	?	M53T/D108N	R151C/+
15a	?	F	+/+	+/+
16a	38	F	+/+	+/+
17a	43	F	+/+	+/+
18a	28	F	+/+	+/+
19a	42	F	+/+	+/+
20a	?	?	+/+	V92M/+
21a	26	F	+/+	D294H/+
22a	21	F	+/+	R151C/+
23a	40	F	+/+	R160W/+
24a	?	F	+/+	R151C/V92M
25a	?	?	+/+	R160W/D294H
26n	NHM	M	+/+	R160W/D294H
27a	23	M	+/+	R151C/R151C
28a	13	M	+/+	R160W/R160W
29n	NHM	M	+/+	R163Q/+

Table 1. : List of primary melanocyte cultures that were established and used in this study. NHM= neonatal human melanocytes; a=adult, and n=neonatal melanocytes; F-female; M=male; +/+=wild-type

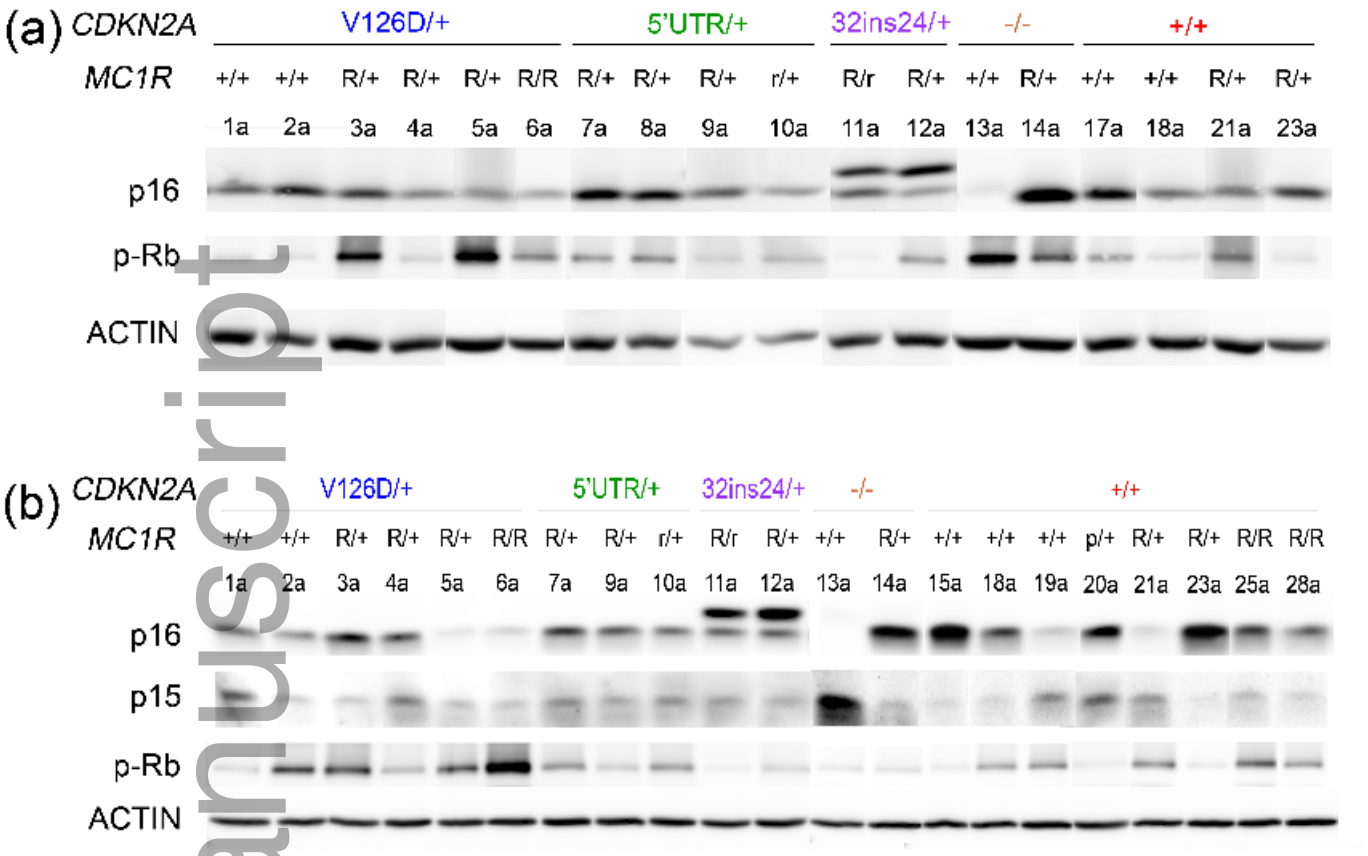

Figure 1

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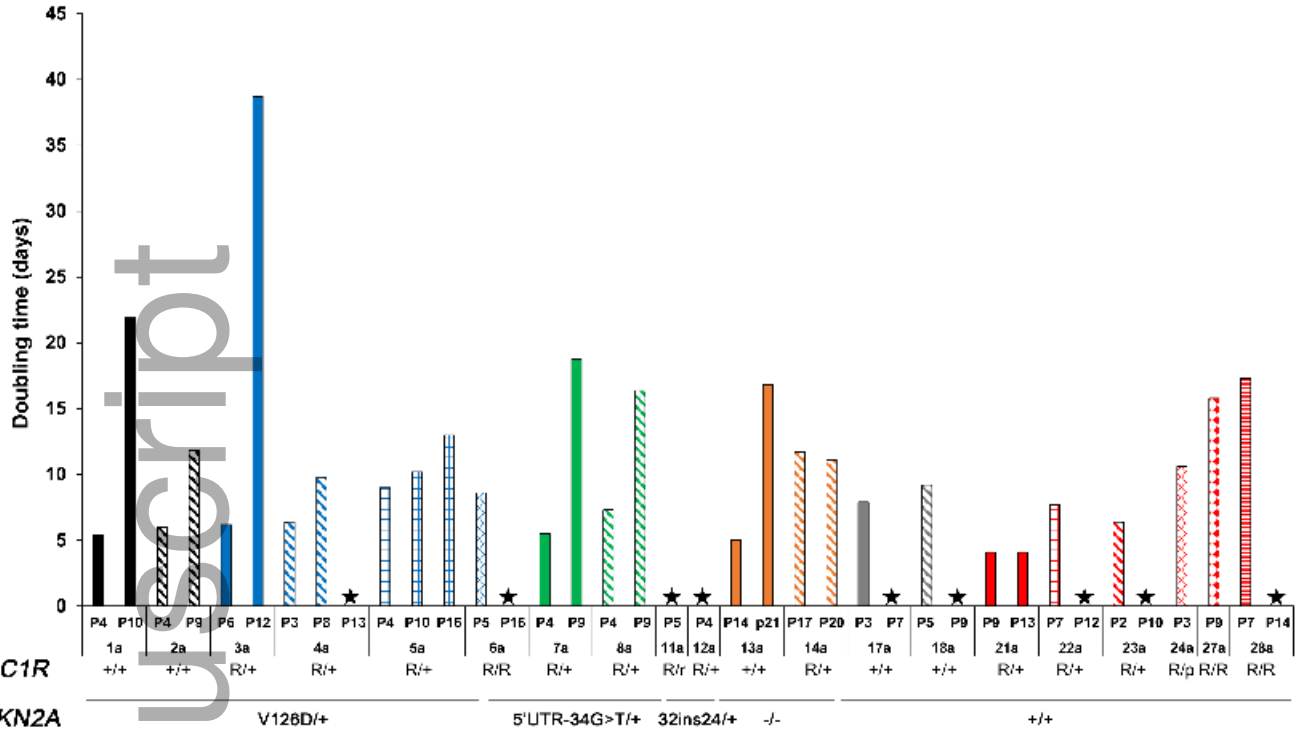

Figure 2

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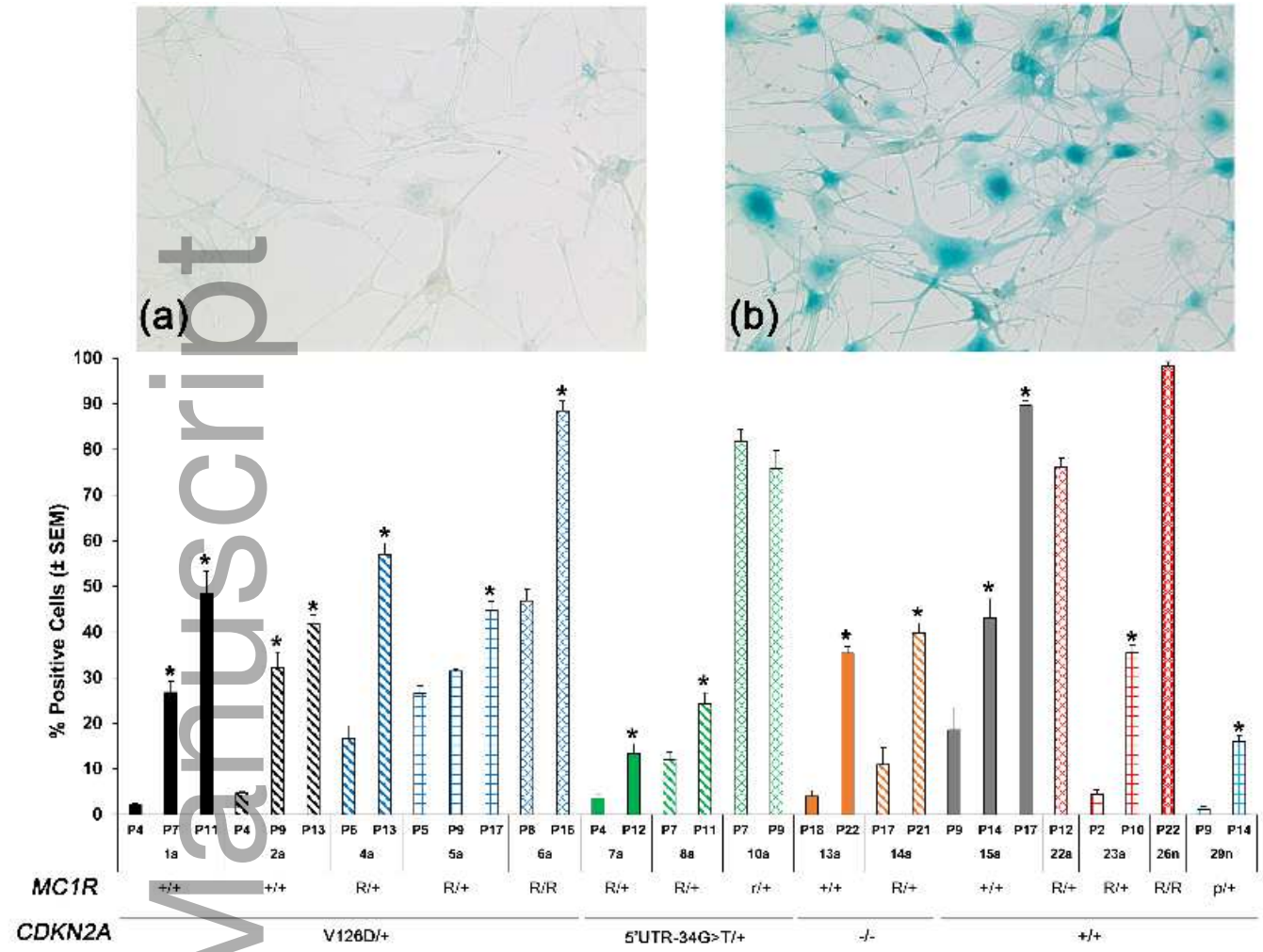

Figure 3

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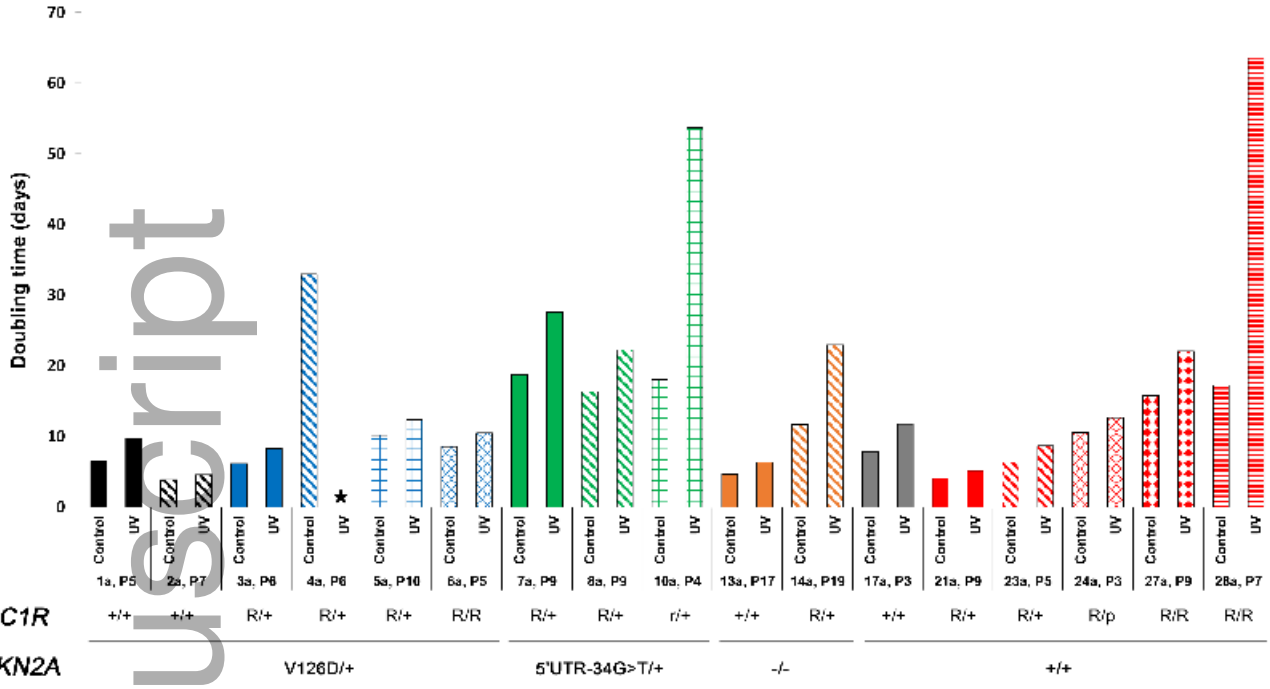

Figure 4

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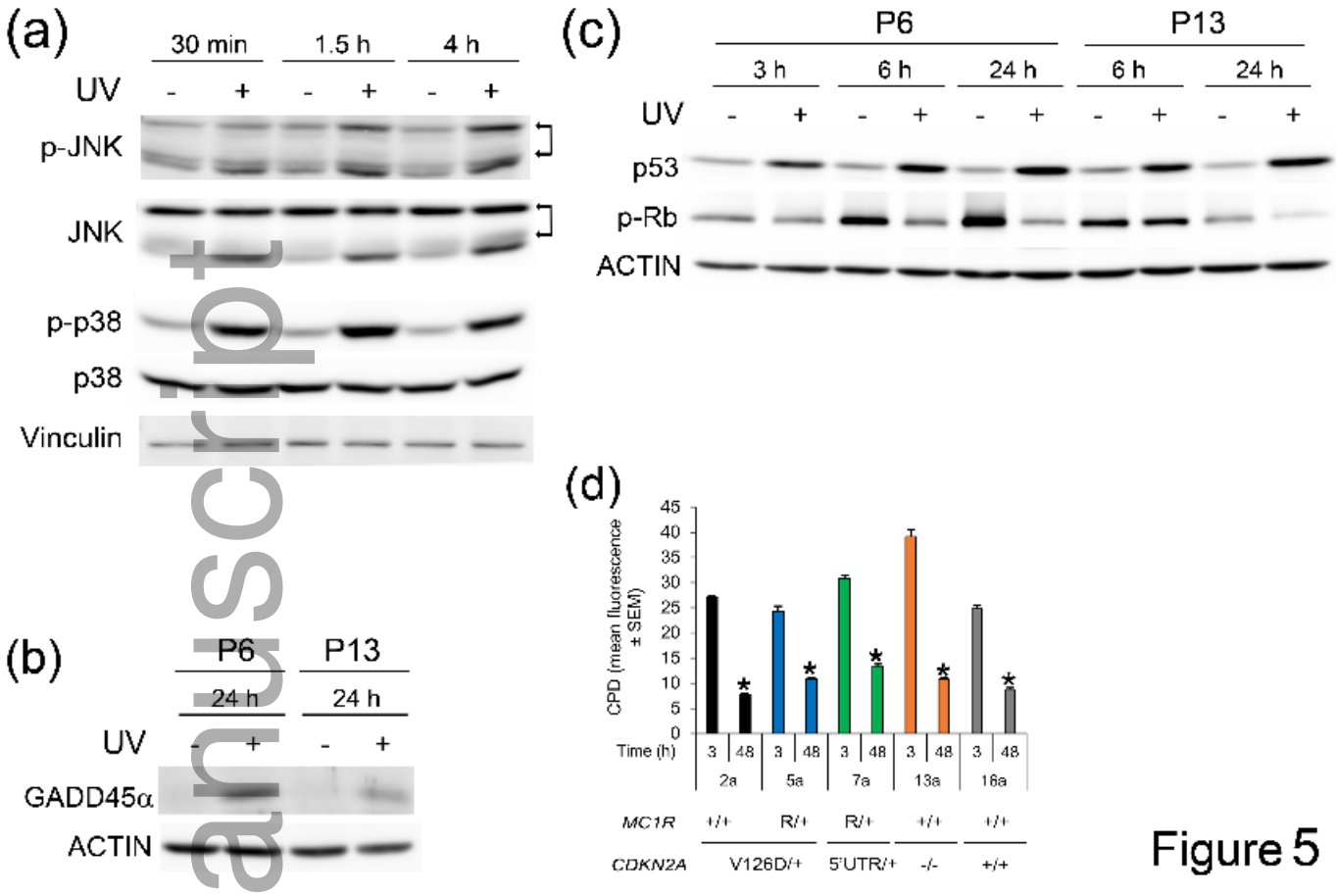

Figure 5

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